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1 **Volatile and fixed composition of sulphite-free white wines obtained**
2 **after fermentation in the presence of chitosan**

3
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7 **RUNNING TITLE: Effects of chitosan on white grape must fermentation**

8
9 **Abstract**

10 Consumers are increasingly interested in healthier wines containing reduced levels or totally absent
11 of sulphites. In the present investigation distinct fermentations of white musts either in the presence
12 of chitosan or sulphur dioxide were carried out in order to compare the volatile and fixed
13 composition of the wines produced, and evaluate the impact of chitosan as an alternative to sulphur
14 dioxide.

15 Chitosan promoted a 24 h extended lag-phase and diminished the titratable acidity of wines by
16 about 1 g L⁻¹ as a consequence of the absorption of tartaric and malic acids onto the polymer
17 surface. The volatile composition of wines was analysed at the end of the alcoholic fermentation
18 and then after 12 months of storage in glass bottle. Hexanoic, octanoic and decanoic acids were
19 significantly higher in chitosan added wines, which further contained an increased amount of ethyl
20 and acetate esters. Results demonstrated that, when added before the alcoholic fermentation,
21 chitosan may affect both the acidic and volatile composition of wines, likely due to its polycationic
22 behaviour and interaction with yeast wall constituents. This also suggests that attention to wine
23 acidic balance should be paid before its use in other vinification steps such as must clarification or
24 wine fining.

25

26 **Keywords:** chitosan; volatile compounds; sulphur dioxide; white wine; SPE-GC/MS

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28

29 **1. Introduction**

30 Sulphur dioxide is undoubtedly the most widely used preservative in oenology thanks to its
31 antioxidant and antimicrobial properties, making it essential for the control of undesirable
32 fermentations and oxidative spoilage in white and red wines.

33 In particular, for what concern oxidation, sulphite effectively counteracts both the phenolic and
34 aromatic decay of wines (Bueno, Culleré, Cacho, & Ferreira, 2010; Waterhouse & Laurie, 2006),
35 otherwise resulting in a decreased attractiveness of final products.

36 However, since seventies, the use of sulphite in foods is being questioned because of its
37 allergenicity, which may cause asthma, dermatitis, urticaria, bronchoconstriction, or anaphylaxis in
38 sensitive humans. (Vally, Misso, & Madan, 2009). Further, in the presence of specific contributory
39 factors, sulphites have been linked to the onset of oncogenic processes. (EFSA, 2004; Lee et al.,
40 2002).

41 Studies about efforts to replace sulphites in wines include physical, chemical or biological
42 treatments. (Santos, Nunes, Saraiva, & Coimbra, 2012; Sonni, Cejudo Bastante, Chinnici, Natali, &
43 Riponi, 2009). The prospective efficacy of some of those techniques has been claimed but further
44 investigations are needed because a convincing alternative to sulphites is still waiting to be found.

45 Chitosan is the deacetylated product of chitin, a homopolymer of n-acetyl-glucosamine, extracted
46 from shellfish wastes, insects or fungal sources. It has several applications in food and
47 pharmaceutical industries, agriculture and water purification, due its features like metal chelation,
48 lipid-lower activity, antimicrobial capacity, film-forming properties, multifaceted antioxidant and
49 radical scavenging activities against hydroxyl and superoxide radicals (Dutta, Dutta, & Tripathi,
50 2004; Yen, Yang, & Mau, 2008). Recently, the use of chitosan has been authorized in must and
51 wine for microbial stabilization or metal and protein removal (EU Commission, 2011). In fermented
52 beverages, chitosan can control the growth of *Brettanomyces spp.* yeasts and lactic bacteria, the
53 both known to spoil wines. Intriguingly, some authors found that this polymer can also contrasts the
54 browning onset and phenolic decay generated by both chemical and enzymatic oxidation in wines

55 and fruit juices (Abd & Niamah, 2012; Chinnici, Natali, & Riponi, 2014; Sapers, 1992; Spagna et
56 al., 1996; Spagna, Barbagallo, & Pifferi, 2000), which makes chitosan a potential candidate for SO₂
57 replacement.

58 Chitosan can be used in several steps along the vinification process, from initial must clarification,
59 to final wine stabilization just before bottling. Unprotected (e.g. sulphite-free) white musts are
60 prone to enzymatic oxidation or unwanted yeast and bacterial proliferation, which may drive to
61 early browning development and sluggish fermentations (Bisson, 1999).

62 Interestingly, the addition of chitosan to free-run juices or during fermentation could acts as both an
63 antioxidant and antimicrobial, in this way reproducing the two main functions that sulphites are
64 called upon to play in the very first phases of winemaking. However, very little is known about the
65 influence of the use of this polymer on musts, on fermentation kinetics and on the volatile
66 composition of the obtained wines.

67 The aim of this work was, hence, to study the effects of the fermentative addition of chitosan on
68 fixed and volatile compounds of sulphite-free white wines.

69 Chitosan was added just before yeast inoculation of white musts and resulting wines were evaluated
70 after 12 months of storage in bottles and compared to wines treated with sulphur dioxide in the
71 same step of the production process.

72

73 **2. Material and Methods**

74 *2.1 Chemicals*

75 Pure standards of volatile compounds, internal standard (2-octanol) and potassium metabisulphite
76 were purchased from Sigma-Aldrich (Milano, Italy).

77 Dichloromethane and methanol (SupraSolv) were supplied by Merck (Darmstadt, Germany),
78 absolute ethanol (ACS grade) was obtained from Scharlau Chemie (Sentmenat, Spain), and pure
79 water was obtained from a Milli-Q purification system (Millipore, USA). LiChrolut EN resin for
80 solid-phase extraction (SPE) prepacked in 200 mg cartridges (3 ml total volume) were purchased

81 from Merck (Darmstadt, Germany). Chitosan (low MW, 75-85% deacetylated, product #448869)
82 was obtained from Sigma-Aldrich (Milano-Italy).

83

84 *2.2 Microvinifications*

85 Sulphite-free white musts were obtained at the experimental winery of the University of Bologna,
86 from grapes cv. Trebbiano. Grapes were destemmed, crushed, pressed at 0.9 bars in a bladder press
87 and cold-settled at 4°C for 24 h. The racked must was then filtered with Seitz-Supra EK1 filters
88 from Seitz (Bad Kreuznach, Germany). The analytical parameters of the obtained must were as
89 follow: sugars 205 g L⁻¹; pH 3.05; titratable acidity 6.8 g L⁻¹; total phenolics 107 mg L⁻¹; O.D. 420
90 nm 0.146. Filtered must was placed in two litres laboratory glass fermentors, at room temperature,
91 to start the fermentation. Trials were arranged in triplicate, before yeast inoculation, by adding
92 potassium metabisulphite or chitosan to the musts at dosage of 60 mg L⁻¹ and 1 g L⁻¹ respectively. A
93 further control fermentation (in triplicate) with no additions was also prepared. To avoid microbial
94 contamination and oxygen entrance during fermentation, each fermentor was provided of a glass
95 trap filled with 37% H₂SO₄. A *Saccharomyces cerevisiae* strain already characterized for its low
96 SO₂ production (strain 1042 from University of Bologna – ESAVE collection) (Sonni et al., 2009)
97 was inoculated after the rehydration of about 1.5×10^6 CFU mL⁻¹ into 25 mL of sterilized must in
98 250-mL Erlenmeyer flasks plugged with cotton wool, incubated for 24 h. Fermentations were
99 monitored by following the weight loss of samples. Once the weight loss stopped, chitosan and
100 yeasts lees were left to settle down and the clarified wines were transferred by means of a peristaltic
101 pump (VWR international, Milano, Italy) in 50 mL bottles, without headspace, and stored for 12
102 months at room temperature and in the darkness. Before the filling, air in the bottles was evacuated
103 by a gentle nitrogen stream.

104

105 *2.3 Oenological parameters*

106 All the oenological parameters were determined according to OIV methods (International
107 Organisation of Vine and Wine (OIV), 2015).

108 The pH was determined by using a pH-meter (Mettler Toledo, Spain). The alcoholic strength of
109 wines was determined by using an oenochemical distilling unit (Gibertini, Italy). Total
110 polyphenolics were spectrophotometrically determined (after wine filtration at 0.45 µm with PTFE
111 filters) at 280 nm using an Uvidec 610 spectrophotometer (Jasco, Japan) and results were expressed
112 as mg L⁻¹ of gallic acid (GAE). All the analyses were carried out in duplicate.

113

114 *2.4 Organic acids, sugars and glycerol*

115 Quantification of organic acids, sugars and glycerol was conducted following the procedure
116 described by Chinnici et al. (Chinnici, Spinabelli, Riponi, & Amati, 2005).

117 The HPLC used was a Jasco apparatus (Tokyo, Japan) equipped with a binary pump (PU 1580), a
118 20 µL loop, a Rheodyne valve (Cotati, CA, USA), a photodiode detector (PU MD 910; Tokyo,
119 Japan), and a column oven (Hengoed Mid Glamorgan, UK). The column was a Bio-Rad Aminex
120 HPX 87H (300 mm×7.8 mm), thermostatted at 35 °C. Isocratic elution was carried out with 0.005 N
121 phosphoric acid at flow 0.4 mL/min. All the analyses were carried out in duplicate.

122

123 *2.5 Wine volatile compounds*

124 Volatile compounds were extracted according to the method described and validated by Lopez et al.
125 (López, Aznar, Cacho, & Ferreira, 2002). A 20 ml wine sample was added of 100 µL of a 2-octanol
126 solution at 500 mgL⁻¹ as internal standard and deposited on an Lichrolut EN cartridge previously
127 activated. Analytes were eluted with 5 mL of dichloromethane, and concentrated to a final volume
128 of 200 µL under a stream of pure nitrogen (N₂), prior to GC-MS analysis.

129 The Trace GC ultra apparatus coupled with a Trace DSQ mass selective detector (Thermo Fisher
130 Scientific, Milan, Italy) was equipped with a fused silica capillary column Stabilwax DA (Restek,

131 Bellefonte, PA, USA; 30 m, 0.25mm i.d., and 0.25 μm film thickness). The carrier gas was He at a
132 constant flow of 1.0 mL/min.

133 The GC programmed temperature was: 45 $^{\circ}\text{C}$ (held for 3 min) to 100 $^{\circ}\text{C}$ (held for 1 min) at 3
134 $^{\circ}\text{C}/\text{min}$, then to 240 $^{\circ}\text{C}$ (held for 10 min) at 5 $^{\circ}\text{C}/\text{min}$. Injection was performed at 250 $^{\circ}\text{C}$ in splitless
135 mode and the injection volume was 1 μL . Detection was carried out by electron ionization (EI)
136 mass spectrometry in full scan mode, using ionization energy of 70 eV. Transfer line interface was
137 set at 220 $^{\circ}\text{C}$ and ion source at 260 $^{\circ}\text{C}$. Mass acquisition range was m/z 30-400 and the scanning
138 rate 1 scan s^{-1} .

139 Compounds were identified by a triple criterion: i) by comparing their mass spectra and retention
140 time with those of authentic standards, ii) compounds lacking of standards were identified after
141 matching their respective mass spectra with those present in the commercial libraries NIST 08 and
142 Wiley 7, iii) matching the linear retention index (LRI) obtained under our conditions, with already
143 published LRI on comparable polar columns (Table 1).

144 Quantification of compounds was carried out via the respective total ion current peak areas after
145 normalization with the area of the internal standard. Calibration curves were obtained by duplicate
146 injections of standard solutions, subjected to the above cited extraction procedure, containing a
147 mixture of commercial standard compounds at concentrations between 0.01 to 200 mg L^{-1} , and
148 internal standard at the same concentration as in the samples. The calibration equations for each
149 compound were obtained by plotting the peak area response ratio (target compound/internal
150 standard) versus the corresponding concentration.

151 For compounds lacking reference standards, the calibration curves of standards with similar
152 chemical structure were used.

153 Analyses were done in duplicate and data were collected by means of Xcalibur software (Thermo
154 Fisher Scientific, Milano, Italy)

155

156 *2.6 Statistical analysis*

157 Statistical analysis of the entire dataset was performed using the XLSTAT Software package
158 (Version 2013.2, France). One-way analysis of variance (ANOVA) followed by a post hoc
159 comparison (Tukey's HSD test) and Principal Component Analysis (PCA) were carried out.

160

161 **3. Results and discussion**

162 *3.1 Fermentation and oenological parameters*

163 The evolution of fermentation was monitored checking the weight loss of fermentors. All the
164 fermentations were completed in 10 days, even if the presence of chitosan resulted in initially
165 slower fermentation rates (**Figure 1**). This was somehow expected since chitosan has already been
166 reported to interfere variably on *Saccharomyces* ssp. growth kinetics (Allan & Hadwiger, 1979;
167 Roller & Covill, 1999). In particular, Roller and Covill (Roller & Covill, 1999) found that the
168 effects on *Saccharomyces* spp. cells growth of 0.4 g L⁻¹ soluble chitosan spanned from complete
169 inactivation to a three days delayed lag phase, depending on the strain considered. These differences
170 in fungi responses have been suggested to be linked to the polyunsaturated free fatty acids content
171 of cells plasma membrane. In sensitive fungi, such as *Neurospora crassa* and *Saccharomyces*
172 *cerevisiae*, the high content of polyunsaturated free fatty acids enhances membrane fluidity and
173 permeabilization leading to augmented intracellular oxidative stress because of the chitosan
174 entrance in the plasma (Lopez-Moya & Lopez-Llorca, 2016; Zakrzewska et al., 2007; Zakrzewska,
175 Boorsma, Brul, Hellingwerf, & Klis, 2005). In our case, the fermentation of samples added with 1g
176 L⁻¹ of chitosan showed a 24 hours extended lag phase but, from day 8 and thereafter, their weight
177 loss was similar to SO₂ or control samples (figure 1). This suggests that the strain used in this
178 experiment was able to resume growth to levels comparable to those observed in untreated musts.

179 At the end of fermentation, samples treated with chitosan had a decreased content of organic acids,
180 with consequent higher pH values (augmented by 0.08 units) and lower titratable acidity (lessened
181 by 1.1 g L⁻¹) (**Table 2**). In particular the grape-derived tartaric and malic acids were reduced by
182 about 0.30 g L⁻¹ and 0.50 g L⁻¹ respectively while, in the same wines, succinic acid amount was

183 0.25 g L⁻¹ lesser. The acid binding properties of chitosan had been already claimed and proposed for
184 the treatment of coffee beverages, vegetable or fruit juices (Imeri & Knorr, 1988; Scheruhn, Wille,
185 & Knorr, 1999). This feature is due to the electrostatic interaction between the positively charged
186 amino groups of glucosamine and the anions coming from dissociated acids, whose pKa and
187 hydroxyl content may also play a role (Mitani, Yamashita, Okumura, & Ishii, 1995).

188 Hence, this would be the reason for the diminution in native organic acids during the 10 days of
189 fermentation. Succinic acid, however, does not come from grapes being produced by yeasts during
190 alcoholic fermentation. Its low amount in KT wines could be the result of reduced fermentative
191 excretion and/or the adsorption by chitosan. It still remains unclear whether one or both the
192 phenomena occurred in our samples.

193 Alcohol content, volatile acidity and total phenolics index were not affected by the treatments
194 while, as expected, the bleaching and antioxidant capacities of sulphite resulted in lighter yellow
195 nuances of final wines if compared with control sample (see tab. 2, at O.D. 420 nm parameter). In
196 this respect, Kt and SO₂ samples were not significantly different in color, suggesting that chitosan
197 may have controlled the browning development, as already reported by other authors (Chinnici et
198 al., 2014; Spagna et al., 2000).

199

200 *3.2 Volatile compositions of wines*

201 A list of volatile compounds found in wines before or after storage is reported in table 1. A total of
202 74 volatiles were elucidated while 12 further compounds lacking of standard and published LRI,
203 were tentatively identified based on their mass spectrum (these compounds are flagged with “MS”
204 in the last column of Table 1).

205 Table 3 reports the amounts of the most significant compounds found in wines at the beginning and
206 at the end of bottle storage, grouped as chemical families, which will be separately discussed.

207

208 *3.2.1 Fatty acids*

209 Our results indicate that treatments with chitosan enhanced the synthesis of three of the main
210 medium chain fatty acids (MCFA), hexanoic, octanoic and decanoic acid (Table 3) that, according
211 to sensory studies, can contribute to the aroma of white wines (Ferreira & Felipe, 2011). During
212 winemaking, a mixture of fatty acids are produced, normally classified as short chain (C2-C4),
213 medium chain (C6-C10), long chain (C12-C18) and branched-chain fatty acids. Metabolism of
214 saturated fatty acids produces straight-chain fatty acids (C4-C12) as intermediate products.
215 (Lambrechts & Pretorius, 2000). The final products, mainly C16 and C18 are incorporated into
216 phospholipids, the backbone of cell membranes. The increased contents of MCFA in wines
217 fermented with chitosan may be due to an augmented permeability of yeast membranes caused by
218 the polysaccharide. As already commented, in fact, at wine pH most of the glucosamine units of
219 chitosan are positively charged due to the protonation of amino groups which allows them to
220 interact with the negatively charged components of cell surface (Zakrzewska et al., 2005).

221 This electrostatic interaction induces changes in the properties of membrane thus modifying, among
222 other, the cell permeability (Hadwiger, Kendra, Fristensky, & Wagoner, 1986).

223 Evidences have been given that growing limiting factors, such as an increased membrane
224 permeability, may cause an augmentation in the production of MCFA by the fatty acid synthase
225 complex (Wakil, Stoops, & Joshi, 1983).

226 These C6 to C10 fatty acids at concentrations $< 10 \text{ mg L}^{-1}$ impart mild and complex aroma to wine.
227 However, at levels above 20 mg L^{-1} , their impact on wines becomes negative (Shinohara, 1985). At
228 the end of fermentation, MCFA concentration in all the samples did not exceed that limit, as
229 reported in table 3.

230 Fermentation conducted in the presence of chitosan showed a decrease in isobutyric and pentanoic
231 acid amounts. The former acid is not produced by the fatty acid synthetic pathway, being derived
232 from oxidation of the aldehydes formed during amino acid metabolism (Ugliano & Henschke,
233 2009).

234 Unpaired acids though, are derived from propionyl-CoA likely formed via α -ketobutyric acid, a
235 metabolite in threonine degradation (Guitart, Orte, Ferreira, Peña, & Cacho, 1999). Their reduced
236 contents in KT wines could be, hence, apparently related to a modification of the amino acid
237 metabolism in yeasts.

238 Fatty acids in wines did not change substantially during the 12 months of bottle storage, confirming
239 the relative stability of this class of compounds when stored at room temperature (Garde-Cerdán,
240 Marsellés-Fontanet, Arias-Gil, Ancín-Azpilicueta, & Martín-Belloso, 2008).

241

242 3.2.2 Esters

243 Volatile esters produced during alcoholic fermentation are of great interest, because of their key
244 role in the sensorial quality of wines, being responsible of fruitness, candy and perfume-like aroma
245 but also of negative notes like “glue-like” aroma (Lambrechts & Pretorius, 2000; Saerens et al.,
246 2008).

247 Chitosan seemed to enhance the esters production, particularly isoamyl acetate, phenylethyl acetate
248 and medium chain fatty acids (MCFA) ethyl esters, ethyl hexanoate, ethyl octanoate, ethyl
249 decanoate and ethyl 3-hydroxybutanoate (Table 3). For ethyl esters, this done is in direct
250 relationship with MCFA amounts in respective wines as the latter are the substrates and limiting
251 factors for the syntheses of the former (Saerens et al., 2008).

252 Acetate esters are formed through the condensation of higher alcohols with acetyl-CoA catalysed in
253 the cell by alcohol acetyltransferase (ATF) enzymes (Mason & Dufour, 2000). However, in KT
254 samples, results did not show any relationship between higher alcohols and acetate esters
255 production (table 3). The reason for the higher amounts of acetates in KT wines is, thus, not clear
256 but it is worth mentioning that ATF enzymes are regulated by the levels of unsaturated fatty acids
257 (UFA) in the medium and that low concentrations in UFA correspond to higher quantities of acetate
258 esters (Saerens, Delvaux, Verstrepen, & Thevelein, 2010).

259 After alcoholic fermentation, a lesser amount of ethyl lactate, ethyl malate, mono and diethyl
260 succinate was found in KT wines. These compounds comes from the esterification of the respective
261 organic acids, whose lower amount in chitosan-treated wines (table 2) may well justify our results.
262 The presence of sulphites led to enhanced production of ethyl-4-hydroxybutanoate, which could be
263 directly related to higher amounts of γ -butyrolactone in SO₂ added wines (Carrau et al., 2008)
264 As expected, during storage, acetate esters drastically decreased while ethyl esters increased to
265 various extents (table 3) in accordance with previous findings (Saerens et al., 2008).
266 In particular, ethyl esters of organic acids significantly raised in concentration after 12 months of
267 storage, and the presence of SO₂ further contributed in promoting their generation as already stated
268 by other authors (Garde-Cerdán et al., 2008)

269

270 3.2.3 Alcohols

271 Together with acids and esters, alcohols are a further important class of yeast-derived volatile
272 compounds in wines, since they play a considerable role in wine aroma (Nykänen, 1986). At the
273 end of fermentation, there were no significant differences in total alcohols content among samples
274 even if differences for some volatiles were found.

275 Isobutyl alcohol and 3-methyl-1-butanol amounts were higher in SO₂ added wines, confirming
276 previous results that postulated that the presence of SO₂ during fermentation favours a prompt
277 consumption of amino acids (Herraiz, Martin-Alvarez, Reglero, Herraiz, & Cabezudo, 1989; Sonni
278 et al., 2009).

279 Quite surprisingly, however, other alcohols deriving from amino acids, such as 2-phenylethanol and
280 4-hydroxybenzenethanol, were not affected by the presence of SO₂, the reason for this behaviour
281 remaining unclear.

282 Sulphites affected the amount of 3-ethoxy-1-propanol which, as already consistently reported
283 (Herraiz et al., 1989; Sonni et al., 2009), is produced in lower quantities in the presence of SO₂.

284 For what concern chitosan, its pre-fermentative addition seemed not to have a considerable
285 influence on alcohols contents, except for the lower levels of isobutyl alcohol and 3-methylthio-1-
286 propanol, the both deriving from amino acid metabolism. This finding may be related to a reduced
287 amino acid availability in musts due to the protein binding features of chitosan (Chatterjee,
288 Chatterjee, Chatterjee, & Guha, 2004).

289 After 12 months of storage, total amount of alcohols in wines increased mostly due to 3-methyl-1-
290 butanol and 2-phenetyl alcohol, without notable differences among samples. Most of the volatile
291 compounds remained unchanged in quantity except 3-methylthio-1-propanol, benzyl alcohol and 4-
292 hydroxy benzenethanol that decreased similarly to what has been already observed in previous
293 works (Garde-Cerdán et al., 2008)

294

295 3.2.4 Other compounds

296 In wine, acetylation occurring between acetaldehyde and glycerol gives raise to heterocyclic
297 compounds such as 1,3-dioxane and 1,3-dioxolane isomers. These compounds, with herbaceous or
298 green olfactory nuances, have been reported to increase in content during wine conservation and
299 aging and have been proposed as markers of Madeira wine ages (Câmara, Marques, Alves, & Silva
300 Ferreira, 2003). Results showed that the amounts of 1,3-dioxanes and 1,3-dioxolane increased
301 drastically during the conservation in bottle but, in sulphite added wines this phenomenon was
302 observed to a significantly lesser extent. This is due to the quenching of acetaldehyde by SO₂ that
303 prevent the reaction with glycerol (Da Silva Ferreira, Barbe, & Bertrand, 2002).

304 Furans are another class of heterocyclic compounds in wine. They mainly originate from
305 monosaccharides that, in acidic medium, degrade via enolization and subsequent dehydration or
306 react with amino acids following the Maillard chemistry (Belitz, Grosch, & Schieberle, 2009).

307 Their presence usually increases with time and is related to sugars level in wine. Table 3 confirms
308 the general augmentation of furanic compounds during storage, in particular for furfural, ethyl 5-

309 oxotetrahydrofuran-2-furancarboxylate and hydroxymethylfurfural that, complessly, tended to be
310 higher in SO₂ samples.

311

312 *3.3 PCA Analysis of volatile compounds*

313 Figure 2 shows the results of the application of PCA (Principal Component Analysis) to the entire
314 dataset of wines volatile compounds. In that figure, for the sake of clarity, only the variables with
315 the highest contribution to the total variance have been plotted. The first component, which explains
316 51.47% of variance, clearly discriminates the samples based on the storage time. On this
317 component, samples at bottling are located in the left quadrants, where the highest variance is due
318 to N-acetyltyramine, isoamyl acetate and 2-hexanol. On the right side, the wines stored for 12
319 months are distinguishable for their content in ethyl esters of succinic, malic and lactic acids.
320 Principal component 2 (31.29% of explained variance) produced a clear separation between KT and
321 the others samples (Control and SO₂) due to the contribution of hexanoic and octanoic acids and
322 ethyl hexanoate, higher in KT wines, and γ -butyrolactone, isobutyric and pentanoic acids which
323 characterized all the samples not containing chitosan.

324

325 **4. Conclusions**

326 The overall results demonstrated chitosan may affect the fermentation and composition of sulphite-
327 free musts. When present all along the fermentation, chitosan may interact with yeasts, delaying
328 the lag phase, and with organic acids, producing a decrease in total acidity. This fact should be
329 taken into consideration even in the case of its use for musts clarification or during the stabilization
330 steps of wines.

331 Concerning the volatile compounds, KT wines had higher concentrations of medium chain fatty
332 acids and related ethyl esters, probably due to the alteration of cell permeability and subsequent
333 perturbation of the fatty acids synthase complex.

334 Except some compounds deriving from amino acids metabolism, alcohols were less affected by the
335 addition of the polysaccharide. Furthermore, differences in volatile composition were maintained
336 over a 12 months storage time. Further investigations are currently being carried out at a semi-
337 industrial scale, which may permit, together with the phenolic characterization, the sensory
338 evaluation of sulphite-free wines fermented in the presence of chitosan.

339

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342

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465

466 **Figure Captions**

467

468 Figure 1: Weight loss of fermentors during the fermentation

469

470 Figure 2: Principal component analysis. Plot of the samples scores and the variables loadings in the
471 plane defined by the first two principal components, at bottling (, gray labels) and after 12 months
472 of storage (black labels). Samples labels: \triangle Control; \circ SO₂; \square KT;

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Table 1

tR (min)	Compound	LRI	Identification ^a
5.04	ethyl 2-methylbutyrate	1078	Std, MS, LRI
5.39	ethyl isovalerate	1090	Std, MS, LRI
5.78	isobutyl alcohol	1104	Std, MS, LRI
6.74	isoamyl acetate	1127	Std, MS, LRI
7.19	n-butanol	1138	Std, MS, LRI
9.44	3-methyl-1-butanol	1194	Std, MS, LRI
10.28	ethyl n-caproate	1221	Std, MS, LRI
11.63	ethyl pyruvate	1265	Std, MS, LRI
12.00	methyl lactate	1281	MS, LRI
12.82	2-hexanol	1304	Std, MS, LRI
13.03	4-methyl-1-pentanol	1309	Std, MS, LRI
13.44	3-methyl-2-buten-1-ol	1319	Std, MS, LRI
13.51	3-methyl-1-pentanol	1321	Std, MS, LRI
14.19	ethyl lactate	1339	Std, MS, LRI
14.52	n-hexanol	1348	Std, MS, LRI
14.84	4-hydroxy-4-methyl-2-pentanone	1357	Std, MS, LRI
14.92	4-methyl-1,3-oxathiolane	1359	MS
15.35	3-ethoxy-1-propanol	1370	Std, MS, LRI
15.72	3-hexen-1-ol	1380	Std, MS, LRI
16.14	nonanal	1391	Std, MS, LRI
17.30	ethyl 2-hydroxy-isovalerate	1421	Std, MS, LRI
17.74	ethyl octanoate	1432	Std, MS, LRI
18.05	5-methyltetrahydro-2-furanyl-methanol	1440	MS, LRI
18.11	2-ethyl-2-methylbutanoic acid	1441	MS
19.03	Furfural	1464	Std, MS, LRI
20.19	cis-5-hydroxy-2-methyl-1,3-dioxane	1493	MS, LRI
20.36	2-mercaptoethanol	1498	Std, MS, LRI
21.05	ethyl-3-hydroxybutyrate	1514	Std, MS, LRI
21.36	2-methyl-3-thiolanone	1522	MS, LRI
21.47	2-(methylthio)ethanol	1524	Std, MS, LRI
22.89	1,3-Dioxolan-2-one	1558	MS
23.07	isobutyric acid	1563	Std, MS, LRI
23.80	propylene glycol	1580	Std, MS, LRI
23.93	ethyl 3-hydroxypropionate	1583	MS
24.35	trans-4-hydroxymethyl-2-methyl-1,3 dioxolane	1593	MS
24.94	γ -butyrolactone	1616	Std, MS, LRI
25.08	n-butyric acid	1623	Std, MS, LRI
25.23	ethyl decanoate	1631	Std, MS, LRI
25.35	N-ethyl acetamide	1637	MS, LRI
26.03	2-furanmethanol (furfuryl alcohol)	1672	Std, MS, LRI
26.25	pentanoic acid	1683	MS, LRI
26.44	diethyl succinate	1693	Std, MS, LRI
27.48	3-methylthio-1-propanol	1733	Std, MS, LRI
28.08	4-hydroxy-2-butanone	1754	MS
28.99	2-hydroxy-methyl ester benzoic acid = methyl salicylate	1787	MS, LRI
29.19	2,7-dimethyl-4,5 octandiol	1794	MS
29.24	ethylphenyl acetate	1796	Std, MS, LRI
29.79	ethyl 4-hydroxybutanoate	1822	Std, MS, LRI
30.01	2-phenylethyl-acetate	1833	Std, MS, LRI
30.11	trans-5-hydroxy-2-methyl-1,3-dioxane	1837	MS, LRI
30.16	4-methyl-2-pentanoic acid	1840	MS
30.76	hexanoic acid	1869	Std, MS, LRI
31.36	N-(3-methylbutyl)acetamide	1899	MS, LRI
31.45	benzyl alcohol	1902	Std, MS, LRI
31.98	ethyl 3-methylbutyl butanedioate	1921	MS, LRI
32.33	2-phenylethanol	1933	Std, MS, LRI
32.86	cinnamyl nitrile	1951	MS
33.35	benzyl oxytridecanoic acid	1967	MS, LRI
34.07	2H-piran-2,6 (3H)-dione	1992	MS
34.63	1H-Pyrrole-2-carboxaldehyde	2017	Std, MS, LRI
34.85	pantolactone	2029	Std, MS, LRI
34.97	diethyl malate	2035	Std, MS, LRI
35.32	octanoic acid	2053	Std, MS, LRI
37.30	N-acetylglycine ethyl ester	2170	MS
37.32	diethyle 2-hydroxypentanedioate	2172	MS
38.03	4-vinyl-2-methoxy-phenol	2213	Std, MS, LRI
38.82	ethyl 5-oxotetrahydrofuran-2-furancarboxylate	2250	MS, LRI
39.17	3-hydroxy-4-phenyl-2-butanone	2267	MS, LRI
39.31	decanoic acid	2274	Std, MS, LRI
39.39	ethyl 2-hydroxy-3-phenylpropanoate	2278	Std, MS, LRI
39.76	3,5-dihydroxy-2-methyl-4H-pyran-4-one	2295	MS, LRI
40.20	glycerin	2313	Std, MS, LRI
40.33	diethyl tartrate	2318	Std, MS, LRI
41.33	ethyl hydrogen succinate	2355	Std, MS, LRI
41.55	4-vinyl phenol	2364	Std, MS, LRI
42.53	2-furancarboxylic acid	2401	Std, MS, LRI
42.92	dodecanoic acid	2427	Std, MS, LRI
43.19	ethyl hydrogen fumarate	2445	MS, LRI
43.50	α -(phenylmethyl) benzeneethanol	2466	Std, MS
44.17	5-(hydroxymethyl)-2-furancarboxaldehyde	2514	Std, MS, LRI
44.25	benzenacetic acid	2521	Std, MS, LRI
46.20	tetradecanoic acid	2673	Std, MS, LRI
48.22	3,4-dimethoxyphenylalanine	2759	MS, LRI
49.39	n-hexadecanoic acid	2803	Std, MS, LRI
50.16	N-acetyltyramine	2840	Std, MS, LRI
50.73	1-H-indole-3-ethanol	2867	Std, MS, LRI
51.77	4-hydroxy-benzenethanol	2944	Std, MS, LRI

Table 1: List of identified compounds. ^a identification assignment: Std = comparing mass spectra, LRI and retention times with pure compounds, MS = by comparing mass spectra with NIST 08 and Wiley 7 spectral database, LRI = matching LRI on comparable polar columns (taken from the following publicly available databases: <https://pubchem.ncbi.nlm.nih.gov/>; <https://www.nist.gov/srd/>; <http://www.flavornet.org/flavornet.html>)

Table 2

	Control	SO ₂	KT
Alcohol (% v/v)	12.07 a	11.99 a	11.97 a
Titrateable Acidity (g L ⁻¹)	6.52 a	6.23 ab	5.25 b
Volatile Acidity (g L ⁻¹)	0.39 a	0.36 b	0.42 a
pH	3.11 b	3.11 b	3.19 a
Total SO ₂ (mg L ⁻¹)	1.92 a	48.7 b	2.56 a
Reducing sugars (g L ⁻¹)	< 2.0 a	< 2.0 a	< 2.0 a
Total phenolics (mg L ⁻¹)	42.3 a	42.3 a	40.7 a
O. D. 420 nm	0.092 a	0.082 b	0.085 ab
Citric acid (g L ⁻¹)	0.20 a	0.19 a	0.18 a
Tartaric acid (g L ⁻¹)	2.94 a	3.03 a	2.67 b
Malic acid (g L ⁻¹)	2.23 a	2.14 a	1.68 b
Lactic acid (g L ⁻¹)	0.18 a	0.23 a	0.18 a
Succinic acid (g L ⁻¹)	0.95 a	0.93 a	0.69 b
Acetic acid (g L ⁻¹)	0.36 a	0.39 a	0.41 a
Glycerol (g L ⁻¹)	9.37 a	9.74 a	9.30 a

Table 2: Enological parameters of wines at the end of alcoholic fermentation
 In the same row, different letters indicate significant differences according to Tukey's test ($p < 0.05$). $n = 3$.

Table 3

	Wines					
	End of fermentation			12 months of storage		
	Control	SO ₂	KT	Control	SO ₂	KT
	Acids					
isobutyric acid	4.04 ^a	3.70 ^a	1.94 ^b	3.42 ^a	2.93 ^a	1.49 ^b
n-butyric acid	0.28 ^b	0.31 ^b	0.35 ^a	0.18 ^c	0.25 ^b	0.30 ^a
pentanoic acid	3.55 ^a	3.53 ^a	2.03 ^b	3.47 ^a	3.44 ^a	1.67 ^b
hexanoic acid	3.58 ^b	3.67 ^b	6.19 ^a	3.52 ^b	3.62 ^b	6.54 ^a
octanoic acid	3.84 ^b	3.85 ^b	7.08 ^a	3.27 ^b	3.32 ^b	6.80 ^a
decanoic acid	1.49 ^b	1.26 ^b	5.33 ^a	1.16 ^b	1.02 ^b	3.77 ^a
dodecanoic acid	0.20 ^a	0.21 ^a	0.18 ^a	0.05 ^b	0.05 ^b	0.10 ^a
benzenacetic acid	0.13 ^b	0.22 ^a	0.06 ^c	0.03 ^b	0.09 ^a	0.05 ^b
<i>Total acids</i>	<i>17.11^b</i>	<i>16.75^b</i>	<i>23.15^a</i>	<i>15.09^b</i>	<i>14.72^b</i>	<i>20.72^a</i>
	Esters					
isoamyl acetate	1.16 ^b	1.04 ^b	1.64 ^a	0.34 ^a	0.36 ^a	0.33 ^a
ethyl hexanoate	0.25 ^b	0.29 ^b	0.65 ^a	0.40 ^b	0.36 ^b	0.75 ^a
ethyl pyruvate	0.06 ^a	0.06 ^a	0.06 ^a	0.13 ^b	0.19 ^a	0.10 ^b
methyl lactate	0.05 ^b	0.03 ^b	0.08 ^a	n.d.	n.d.	n.d.
ethyl lactate	1.08 ^b	1.30 ^a	0.86 ^c	3.92 ^a	3.39 ^b	3.44 ^b
ethyl octanoate	0.10 ^b	0.20 ^b	0.44 ^a	0.70 ^b	0.54 ^b	1.33 ^a
ethyl-3-hydroxybutyrate	0.12 ^b	0.07 ^b	0.17 ^a	0.12 ^b	0.16 ^a	0.16 ^a
ethyl decanoate	0.00 ^b	0.05 ^b	0.16 ^a	0.10 ^b	0.07 ^b	0.42 ^a
diethyl succinate	0.18 ^a	0.20 ^a	0.14 ^b	6.39 ^{a,b}	7.45 ^a	4.48 ^b
methyl salicylate	0.04 ^a	0.02 ^a	0.04 ^a	n.d.	n.d.	n.d.
ethyl 4-hydroxybutanoate	2.64 ^b	3.33 ^a	1.09 ^c	0.05 ^{a,b}	0.12 ^a	0.01 ^b
2-phenylethyl acetate	0.87 ^b	0.93 ^b	2.10 ^a	0.12 ^b	0.14 ^b	0.36 ^a
diethyl malate	0.40 ^a	0.41 ^a	0.28 ^b	6.89 ^b	11.43 ^a	7.15 ^b
diethyl tartrate	n.d.	n.d.	n.d.	0.67 ^b	1.17 ^a	0.40 ^b
ethyl hydrogen succinate	2.77 ^a	2.85 ^a	2.11 ^a	11.73 ^a	13.57 ^a	14.71 ^a
<i>Total esters</i>	<i>9.71^a</i>	<i>10.79^a</i>	<i>9.83^a</i>	<i>31.59^a</i>	<i>38.98^a</i>	<i>33.64^a</i>
	Alcohols					
Isobutyl alcohol	20.27 ^b	28.23 ^a	13.46 ^c	27.54 ^a	20.46 ^b	14.83 ^c
2-hexanol	0.02 ^c	0.08 ^a	0.05 ^b	0.08 ^a	0.07 ^a	0.08 ^a
3-methyl-1-butanol	30.64 ^b	40.12 ^a	30.59 ^b	55.27 ^a	45.43 ^a	55.81 ^a
2-hexanol	0.05 ^a	0.05 ^a	0.05 ^a	0.03 ^a	0.03 ^a	0.03 ^a
4-methyl-1-pentanol	0.00 ^c	0.01 ^b	0.02 ^a	0.00 ^b	0.01 ^a	0.01 ^a
n-hexanol	0.09 ^a	0.10 ^a	0.07 ^b	0.08 ^a	0.09 ^a	0.06 ^b
3-ethoxy-1-propanol	0.19 ^a	0.11 ^b	0.17 ^a	0.18 ^a	0.09 ^c	0.15 ^b
3-hexen-1-ol	0.03 ^b	0.03 ^a	0.03 ^{a,b}	0.03 ^a	0.03 ^a	n.d.
3-methylthio-1-propanol	1.06 ^a	1.17 ^a	0.41 ^b	0.63 ^a	0.65 ^a	0.27 ^b
Benzyl alcohol	0.20 ^{a,b}	0.29 ^a	0.11 ^b	0.10 ^a	0.12 ^a	0.09 ^a
2-mercaptoethanol	n.d.	0.01 ^a	n.d.	n.d.	n.d.	n.d.
Phenethyl alcohol	38.97 ^a	38.36 ^a	37.40 ^a	63.50 ^a	72.55 ^a	76.03 ^a
4-hydroxy-benzenethanol	20.54 ^a	20.63 ^a	22.88 ^a	14.26 ^a	19.38 ^a	20.91 ^a
<i>Total alcohols</i>	<i>112.08^a</i>	<i>127.20^a</i>	<i>108.25^a</i>	<i>161.69^a</i>	<i>158.89^a</i>	<i>168.26^a</i>
	Others					
cis-5-hydroxy-2-methyl-1,3-dioxane	0.03 ^b	0.05 ^a	0.04 ^a	1.75 ^b	0.87 ^c	3.19 ^a
trans-4-hydroxymethyl-2-methyl-1,3 dioxolane	0.02 ^b	0.10 ^a	0.04 ^b	0.76 ^b	0.44 ^c	1.26 ^a
trans-5-hydroxy-2-methyl-1,3-dioxane	0.04 ^a	0.05 ^a	0.04 ^a	1.64 ^b	1.02 ^c	2.59 ^a
γ-butyrolactone	0.28 ^b	0.37 ^a	0.12 ^c	0.19 ^b	0.26 ^a	0.09 ^c
Furfural	0.07 ^a	0.07 ^a	0.08 ^a	0.12 ^c	0.44 ^a	0.25 ^b
Furfuryl alcohol	0.10 ^a	0.13 ^a	0.12 ^a	0.06 ^a	0.03 ^b	0.07 ^a
4-hydroxy-2-butanone	0.88 ^a	0.76 ^b	0.55 ^c	-0.01 ^b	0.04 ^a	0.05 ^a
ethyl 5-oxotetrahydrofuran-2-furancarboxylate	0.79 ^a	0.86 ^a	0.31 ^b	1.01 ^b	1.61 ^a	0.93 ^b
2-furancarboxylic acid	0.08 ^b	0.17 ^a	0.08 ^b	0.19 ^{a,b}	0.23 ^a	0.13 ^b
5-(hydroxymethyl) 2-furancarboxaldehyde	n.d.	n.d.	n.d.	0.73 ^b	0.95 ^{a,b}	1.32 ^a
N-acetyltyramine	0.10 ^b	0.14 ^a	0.13 ^a	n.d.	n.d.	n.d.
<i>Total others</i>	<i>2.40^a</i>	<i>2.69^a</i>	<i>1.51^b</i>	<i>6.43^b</i>	<i>5.90^b</i>	<i>9.87^a</i>

Table 3. Concentration of the quantified volatile compounds (mg L⁻¹) in wines at the end of the alcoholic fermentation and after 1 year of bottle storage.

In the same row, different letters indicate significant differences according to Tukey's test (p<0.05). n=3.

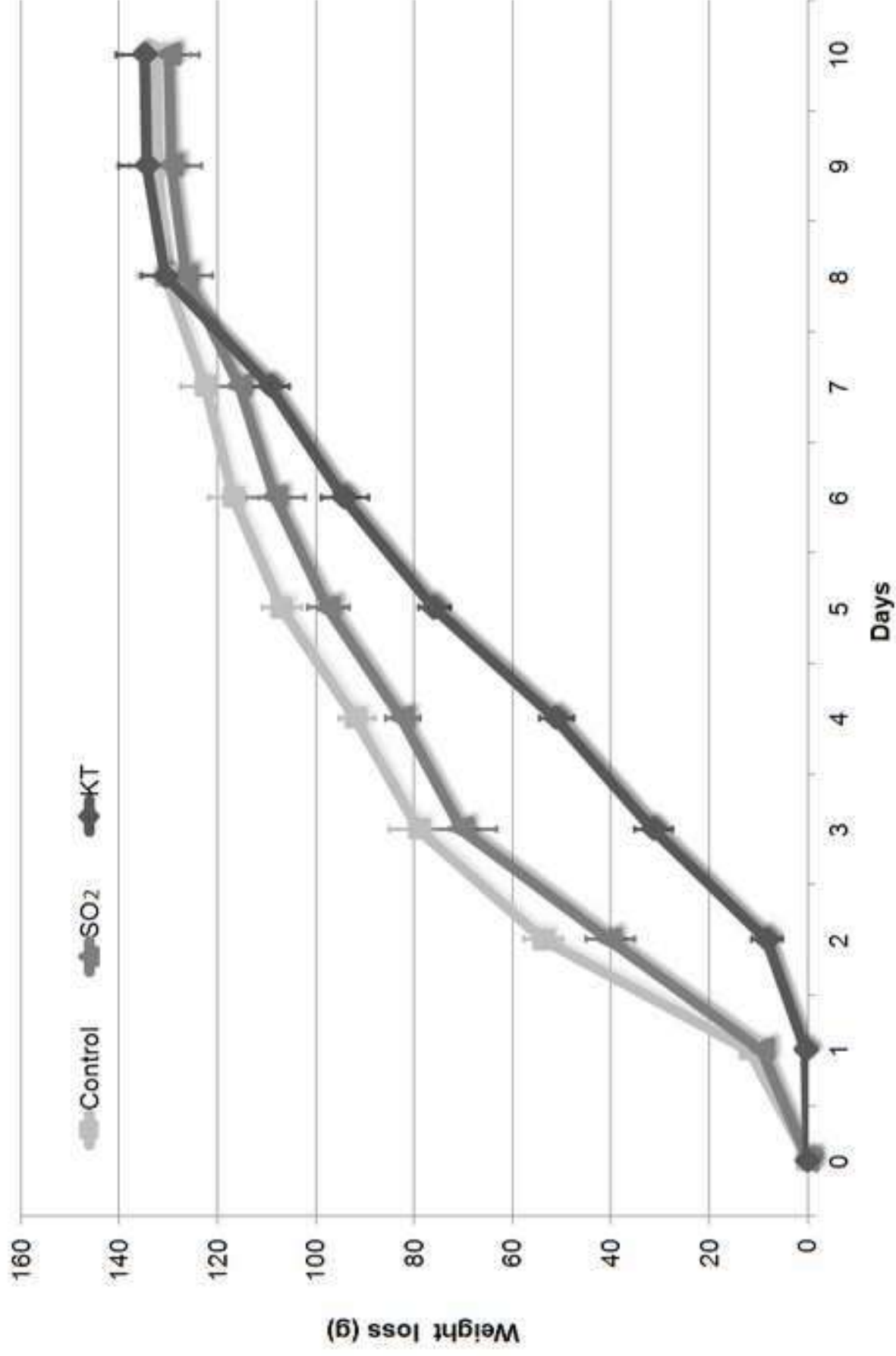


Figure 1
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Figure 2

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